Smc5/6: a link between DNA repair and unidirectional replication?

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Smc5/6: a link between DNA-repair and unidirectional replication?

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Preface

Of the three structural maintenance of chromosome (SMC) complexes, two regulate chromosome dynamics. The third, Smc5/6, functions in homologous recombination and the completion of DNA replication. The literature suggests that Smc5/6 coordinates DNA repair, in part through post-translational modification of uncharacterised target proteins that can dictate their subcellular localization. Smc5/6 functions to establish DNA damage-dependent cohesion. A nucleolar-specific Smc5/6 function has been proposed because Smc5/6 complex yeast mutants display penetrant phenotypes of rDNA instability. rDNA repeats are replicated unidirectionally. Here we propose that unidirectional replication, combined with global Smc5/6 complex functions can explain the apparent rDNA specificity.

Introduction

SMC complexes regulate high order chromosome structure. Cohesin maintains the link between replicated sister chromosomes that is essential for equal mitotic segregation. Condensin compacts chromatin prior to mitosis. Smc5/6 complex plays a poorly characterised role in DNA repair. The extensive architectural, structural and sequence similarity between the three SMC complexes suggest common modes of action. There is also an intriguing conservation of domain architecture with bacterial Sbc nucleases and the Mre11-Rad50-Nbs1 (MRN) complex (see Ref.[1]). Each SMC complex is based on a heterodimer of two SMC proteins and includes between two and six non-SMC subunits. The SMC subunits have the characteristic structure of two globular heads separated by an extensive coiled-coil region interrupted by a central ‘hinge’. Each SMC molecule folds back on itself at this hinge, bringing the globular domains together to form an ATP-binding and hydrolysis site. This consequently presents an extensive coiled-coil tail terminating at the hinge. The heterodimers associate tightly through these hinge regions, whereas the globular ATPase domains are bridged together by non-SMC subunits.

This characteristic structure allows Cohesin (which comprises Smc1 and Smc3) to form a ring that encircles sister chromatids which can be opened during mitosis by cleavage of the kleisin subunit bridging the globular heads (see Box 1). While the conservation of a kleisin-like subunit and of coiled-coil domain length is could reflect a common mode of chromatin association, there is no evidence that

Condensin (which comprises Smc2 and Smc4) or Smc5/6 form rings or have cleavable subunits..

Smc5/6 complex (which comprises Smc5, Smc6 and subunits Nse1-6) was initially identified when *smc6* complemented a DNA-damage-sensitive fission yeast mutant[2]. Genetic analysis placed the Smc5/6 complex's DNA-repair functions in the homologous recombination (HR) repair pathway and identified an additional essential function related to the repair of spontaneous DNA-replication lesions[2-12]. Here we review the emerging Smc5/6 literature and propose that the consequence of Smc5/6 loss of function may be revealed most readily when replication is unidirectional.

Smc5/6 in repair and replication

Viable hypomorphic (partially functional) *smc5*, *smc6* and *nse* mutants have been used to analyse the DNA-repair functions of Smc5/6[2-9,11,13]. Using a pulse field gel electrophoresis (PFGE) assay it is clear that chromosomes in these mutants are unrepaired following ionising radiation (IR)[8,12,14]. This is indicative of defective HR because the IR-induced DNA fragments are not rejoined.

HR is required to cope with perturbations in DNA replication. Consistent with an HR function, the same hypomorphic Smc5/6 complex mutants fail to separate their DNA at mitosis following replication perturbation[9,12,15,16]. This results in multiple catastrophic mitoses which can be alleviated by concomitant HR loss[15,16]. This is inconsistent with a defect in the strand exchange and ligation stages of HR repair. Instead, it must reflect a defect in later HR functions such as joint molecule resolution, loss of which would results in the accumulation of linked sister chromosomes, preventing their separation at mitosis. Thus, IR-induced damage and replication errors show problems in HR at distinct stages when Smc5/6 complex function is attenuated.

Ambiguous roles in double strand break (DSB) repair. We cannot unambiguously assign one specific HR function to the Smc5/6 complex. Furthermore, complete loss of HR is not lethal in yeast. Concomitant loss of Smc6 and HR does not reverse the inviability resulting from Smc6 loss[3,11], confirming an additional HR-independent function. The necessary use of hypomorphic yeast mutants, and small interfering RNA (siRNA) in mammalian cells, remains a significant limitation in studying Smc5/6; it does not allow a straightforward assignment of function. In mammalian cells siRNA ablation (with >90% efficiency) results in a defect in

recombination between sister chromatids. The authors suggested this could be related to an additional defect in the ability to recruit Cohesin to the sites of DNA double strand breaks[10]. This is supported by observations that Cohesin recruitment to the site of a break[14,17] is Smc5/6 complex dependent[4,10]. Interestingly, *smc6* mutants also cannot support the reestablishment and reinforcement of cohesion on undamaged chromosomes in response to DNA-damage checkpoint signalling[18,19], even though the genome wide association of Cohesin at these undamaged regions is Smc5/6 complex independent[19].

Yeast hypomorphic mutants similarly showed a defect in an indirect assay for recombination between sister plasmids, while retaining apparently normal HR between repeats on the same chromosome arm[4]. However, while Smc5/6 is clearly required for sister chromatid recombination, it cannot be concluded that Smc5/6 function is not required for all HR. The lack of effect on non-sister chromatid HR could reflect a threshold effect (siRNA) or a separation of function phenotype (hypomorphic mutants). One report suggests that a different hypomorphic *smc6* mutant is defective in non-sister chromatid recombination[20]. In *Arabidopsis thaliana*, Smc5/6 complex defects result in decreased recombination between homologous chromosomes[21]. We can conclude that Smc5/6 cooperates with other repair factors, including MRN and Cohesin, to direct repair towards sister chromatids (this limits recombination between separate chromosomes) but cannot conclude this is the only HR function.

How might Smc5/6 complex regulate HR? One Smc5/6 complex subunit, Nse2, is a SUMO ligase[22-24]. In yeast, a catalytic-site mutant defective in ligase activity is viable but shows phenotypes suggestive of an intermediate HR defect[22,24]. In human cells, siRNA knockdown and expression of an siRNA resistant mutant similarly demonstrated the catalytic-site mutant is HR defective[23].

A function in telomere maintenance has been associated with Nse2 SUMO ligase activity[25]. Several telomere-associated proteins were identified as potential substrates and either Smc5/6 ablation or loss of SUMO-modified forms of telomere-binding proteins TRF1 and TRF2 were shown to reduce HR-dependent telomere elongation via the alternative lengthening of telomeres (ALT) pathway (see Box 2). This correlated with loss of telomere recruitment to promyelocytic leukaemia (PML) bodies, a hallmark of ALT activation. Smc5/6 complex was shown to localize to PML bodies, implying a specific role in recruitment of telomeres to such sites via SUMO

modification of telomere-binding proteins. This could indicate that the Smc5/6 complex functions, in part (SUMO ligase function is not essential), to coordinate HR in particular circumstances, in this case via post-translational modification and the subsequent intracellular relocalization of telomere proteins within PML bodies.

Other evidence for regulatory roles in HR. A detailed study of Nse2 function at stalled replication forks demonstrated that Rad51-dependent X-shaped molecules accumulate in the absence of SUMO ligase activity[26]. The authors suggested that Smc5/6 complex-mediated SUMO modification of unknown proteins acts in concert with the Blooms' helicase homologue, Sgs1, to limit the accumulation of pathological cruciform structures when replication forks stall. Similar joint DNA molecules accumulate at collapsed replication forks in fission yeast *smc6* mutants, correlating with the chromosome missegregation phenotype[15]. Again, these data hint at a regulatory role rather than a direct enzymatic function for the Smc5/6 complex in HR and it will be interesting to define the targets and study their localization.

There is a wealth of genetic data, particularly in *Schizosaccharomyces pombe*, that similarly hint at a role for Smc5/6 complex in coordinating DNA repair activities. Brc1, a protein consisting of six consecutive BRCA1 C-terminal (BRCT) motifs, was found as an allele-specific multi-copy suppressor of the *smc6-74* mutant[12]. Two separate *smc6* mutants, *smc6-X* and *smc6-74*, have been extensively characterized in *S. pombe*, but only *smc6-74* is suppressed by multi-copy *brc1*. Suppression is specific to induced DNA damage and requires multiple additional DNA repair functions, including post-replication repair and a variety of DNA repair nucleases[27,28]. The budding yeast homologue of Brc1 (Esc4) forms foci in response to Methyl Methane Sulfonate (MMS)-induced damage and binds to many DNA repair proteins, including Rad55 and Slx4. It has thus been proposed to scaffold repair factors[29-31].

brc1 overexpression likely facilitates the bypass of one or more specific Smc5/6 functions (that are defective in *smc6-74* but not *smc6-X*) by alternative repair processes[27]. If Brc1 overexpression is promoting bypass suppression by affecting the local concentrations of alternative repair proteins, this would be consistent with a regulatory rather than enzymatic repair function being the defining *smc6-74* defect. While we must be cautious of how much weight we apply to such genetic data, taken together, they are consistent with roles in coordinating HR and other repair functions in response to damaged DNA.

rDNA replication and repeat instability

A number of studies have focused on the consequences of Smc5/6 defects for rDNA replication and stability. Like Cohesin and Condensin, Smc5/6 associates with chromatin genome-wide. In unperturbed *S. cerevisiae* cells two modes of recruitment, Scc2-dependent and Scc2-independent, have been identified[14]. Scc2 is the Cohesin loading factor, but the regions of Scc2-dependent Smc5/6 complex enrichment are not identical with Cohesin enrichment sites. Chromatin immunoprecipitation studies have shown that Smc5/6 is enriched on the *S. cerevisiae* rDNA[11], although this is not evident in *S. pombe*[15]. However, in both yeasts, immunofluorescence reveals that a significant fraction of Smc6 localizes to the nucleolus[11,15] which contains ~200 rDNA repeat units (Figure 1A).

In *S. cerevisiae*, rDNA segregation is defective when Smc5/6 function is compromised[11]. This does not explain the essential Smc5/6 function because global chromosomal missegregation and fragmentation are a feature of Smc5/6 ablation in *S. pombe*[5,7,8,32] and, in *S. cerevisiae*, the replacement of the rDNA repeats with RNA polymerase II (pol II) expressed episomal sequences does not rescue lethality[11].

Is rDNA replication specifically at fault? What causes rDNA instability and missegregation in Smc5/6 complex mutants? A recent study suggested that rDNA replication is incomplete when Smc5/6 function is compromised[33]. DNA from metaphase arrested *S. cerevisiae* cells was stretched onto a glass slide following one round of S phase in the presence of BrdU. Antibody staining revealed an increase in BrdU-free gaps, presumed to reflect incomplete rDNA replication, from a baseline of 4.5% in *SMC6*$^{+}$ cells to 8.2% in an *smc6* hypomorph.

However, it is unclear why 4.5% of metaphase-arrested *SMC6*$^{+}$ cells should contain unreplicated gaps in their rDNA when cell viability is >99%. It is unlikely that a twofold increase in these potential replication gaps could accounts for an increase in rDNA missegregation from <5% in *SMC6*$^{+}$ to >60% in Smc5/6 complex mutants. In metaphase-arrested *smc6* mutant cells, replication and recombination intermediates are also increased ~3-fold and programmed replication pause signals (see below) increased ~7-fold. This has been suggested to imply delayed rDNA replication in Smc5/6 complex mutants and contribute to increased missegregation. However, it is again unclear why *SMC6*$^{+}$ metaphase-arrested cells should display significant levels of replication or recombination intermediates. An alternative explanation is that the structures visualized derive from the ~5% of cells that are not

synchronized by the procedure used, and thus do not reflect delayed rDNA replication in either *SMC6*$^{+}$ or Smc5/6 complex mutants.

Unidirectional rDNA replication.

Replication of the rDNA is unidirectional to ensure that transcription and replication do not clash and promote replication fork breakage (see Ref.[34]). Unidirectional replication occurs because the Fob1 protein, which binds to specific sites in the rDNA repeat, induces replication fork barrier (RFB) activity for forks travelling in one direction (leftward fork in Figure 1A). Because less that one in five rDNA origins fire, this results in the majority of the rDNA being replicated unidirectionally.

The unidirectional replication pattern likely provides the mechanism underpinning rDNA repeat homeostasis: when rDNA repeat copy number is appropriate, the silencing protein Sir2 recruits Cohesin to the cohesion-associated region (CAR) within each rDNA repeat (Figure 1B) [34]. This directs rDNA recombination to the sister chromatid, preventing unequal sister chromatid exchange (SCE) or interchromosomal repeat recombination (left panel).

In response to nutrients and intracellular signal a poorly understood series of events results in bidirectional pol II-dependent transcription from the *epro* promoter within the rDNA repeats (located adjacent to the CAR). This results in Cohesin dissociation and a consequent increase in unequal SCE (right panel). Thus, when rDNA copy number dips below, or rises above, a predetermined threshold, an appropriate increase or decrease in rDNA copy number is possible by regulating *epro*-dependent transcription.

The programmed polar RFBs that establish unidirectional replication have been proposed to initiate recombination within the rDNA at a constant rate during each S phase. Normally, these HR events are targeted to the sister chromatid and rapidly and silently resolved. If Cohesin binding at CAR is perturbed by *epro* transcription expansions and contractions will be induced. Thus, regulation of HR outcome (equal v unequal sister chromatid recombination), as opposed to HR initiation, may be the regulated step that maintains rDNA stability.

Consistent with specific control of HR in rDNA, repair protein foci dynamics are altered in the nucleolus[33]. DNA damage caused to the rDNA by exogenous agents such as IR do not result in recombination foci in the nucleolus of *S. cerevisiae*, although MRN and DNA damage checkpoint proteins foci can be visualized. The

parsimonious explanation is that rDNA repair in the nucleolus is rapid, likely requiring less time for homology search and resulting in less processing because of the close proximity of the sister chromatid.

The increase in rDNA instability when Smc5/6 complex function is attenuated is correlated with the appearance of HR protein foci in the nucleolus[33]. The Smc5/6 complex contains a SUMO ligase and SUMO modification of the key HR protein Rad52 is also required to suppress the formation of nucleolar HR protein foci[33]. The circle would have been closed nicely if Rad52 were the target of Nse2 and Rad52-SUMO promoted both nucleolar exclusion of HR protein foci and increased HR-dependent rDNA instability. Unfortunately, Rad52 is not a target of the Smc5/6 complex and the nucleolar HR protein foci that form in cells where Rad52 cannot be SUMO modified do not correlate with in increased rDNA instability. (It was not established if the Smc5/6 complex mutants used were SUMO ligase deficient or if the ligase-defective *nse2* mutant is able to suppress nucleolar HR protein foci).

These data could imply a nucleolar-specific Smc5/6 function that serves to suppress rDNA recombination. However, it is likely erroneous to equate HR protein foci with DNA repair: an accumulation of tagged molecules is required to visualize foci and we do not know if the same number of molecules is required for successful repair of the rDNA. An equally valid assumption is that rDNA repair is rapid because of CAR-dependent Cohesin loading and/or other unknown processes. Compromising general HR with Smc5/6 complex mutants may delay or disrupt repair and allow HR proteins to accumulate sufficiently to be visualised as foci.

The importance of unidirectional replication.

The ability to restart stalled or broken replication forks is of particular importance when two converging forks stop. If a single fork stops, even if it cannot be recovered the problem is resolved by convergence of the oncoming fork (Figure 1C, left). However, in a region of unidirectional replication, a single stopped fork must be recovered by restart or recombination-based replication because no converging fork will be available (Figure 1C, right). Two genomic regions are replicated unidirectionally: the rDNA repeats and the telomeres. It is intriguing that both these regions have been identified as potent readouts of Smc5/6 function.

In *smc6* mutants, loss of unidirectional replication by *fob1*deletion results in a two-fold decrease in rDNA missegregation. Loss of pol I-dependent rDNA

transcription results in a three-fold decrease. Loss of both *fob1* and rDNA transcription abolishes the Smc5/6 complex mutant-dependent rDNA missegregation[33]. Thus programmed or transcription-dependent replication fork stalling are likely initiating this rDNA instability.

Is there an rDNA-specific function for Smc5/6? We propose that the unidirectional nature of rDNA replication is sufficient to emphasize loss of a genome-wide Smc5/6 complex functions. If a single replication fork stops outside of the rDNA or telomeric regions, a converging fork will complete replication. However, if a single fork stops within the rDNA or telomere, it will result in a region of DNA that cannot be replicated. Thus, the rDNA instability and missegragation phenotypes that are prevalent in Smc5/6 complex mutants are consistent with a genome-wide function for Smc6 in fork restart. The phenotypes are accentuated because of unidirectional replication.

It is not possible to determine directly if the rDNA phenotypes of Smc5/6 complex mutants result from a global increase in replication forks stopping, the same number of stopped forks being prone to a global failure to restart, or misregulation of HR following restart. Each possibility (or a combination of them) would, because of unidirectional replication, result in an increased repeat instability and increased rDNA missegregation that would not be so apparent in bidirectionally replicated regions. Occam's razor[35] posits that when two theories make the same predictions, the simpler one is better. Appling this principle, there is no reason to propose that Smc5/6 specifically regulates rDNA replication or plays an rDNA-specific role in HR regulation.

Conclusion

We can confidently assume a genome-wide role for Smc5/6 in coordinating HR and resolving joint molecules in yeast and higher eukaryotes. The data indicate that the Smc5/6 complex may, likely in part, regulate recombination and recombination outcomes by affecting post-translational protein modification of largely unknown targets. These modifications may determine sub-cellular protein localization, such as that seen in ALT positive human cells, and thus their function. A global role for the Smc5/6 complex is sufficient to explain the prevalence of phenotypes in the yeast rDNA when we take into account the highly regulated nature of HR in the rDNA and the fact that unidirectional replication will reveal otherwise common defects more

avidly. The Smc5/6 complex has an intriguing architectural similarity to Cohesin, which may encompass two sister chromatids, and Condensin which remodels chromatin for mitosis. Exactly what this similarity means in respect of Smc5/6 function remains an intriguing question: perhaps the Smc5/6 complex promotes or stabilizes specific chromatin conformations to facilitate various aspects of DNA repair.

Word count: 2992.

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Box 1. The family of SMC complexes

Smc5/6 complex contains eight subunits, Smc5 and Smc6 plus six non-Smc subunits (Nse1-6)[13,24]. Nse1-4 are essential, whereas Nse5 and Nse6 are inessential but are required for the repair functions[13]. An additional loosely associated subunit, Rad60, is required for both DNA-repair and the essential function of the Smc5/6 complex[36,37]. The complex is closely related to Cohesin and Condensin. The N- and C-terminal globular domains of each SMC subunit self-associate to generate an ATPase[1,38]. SMC complexes are distantly related to the Rad50 complex and various bacterial complexes involved in condensation and chromosome segregation.

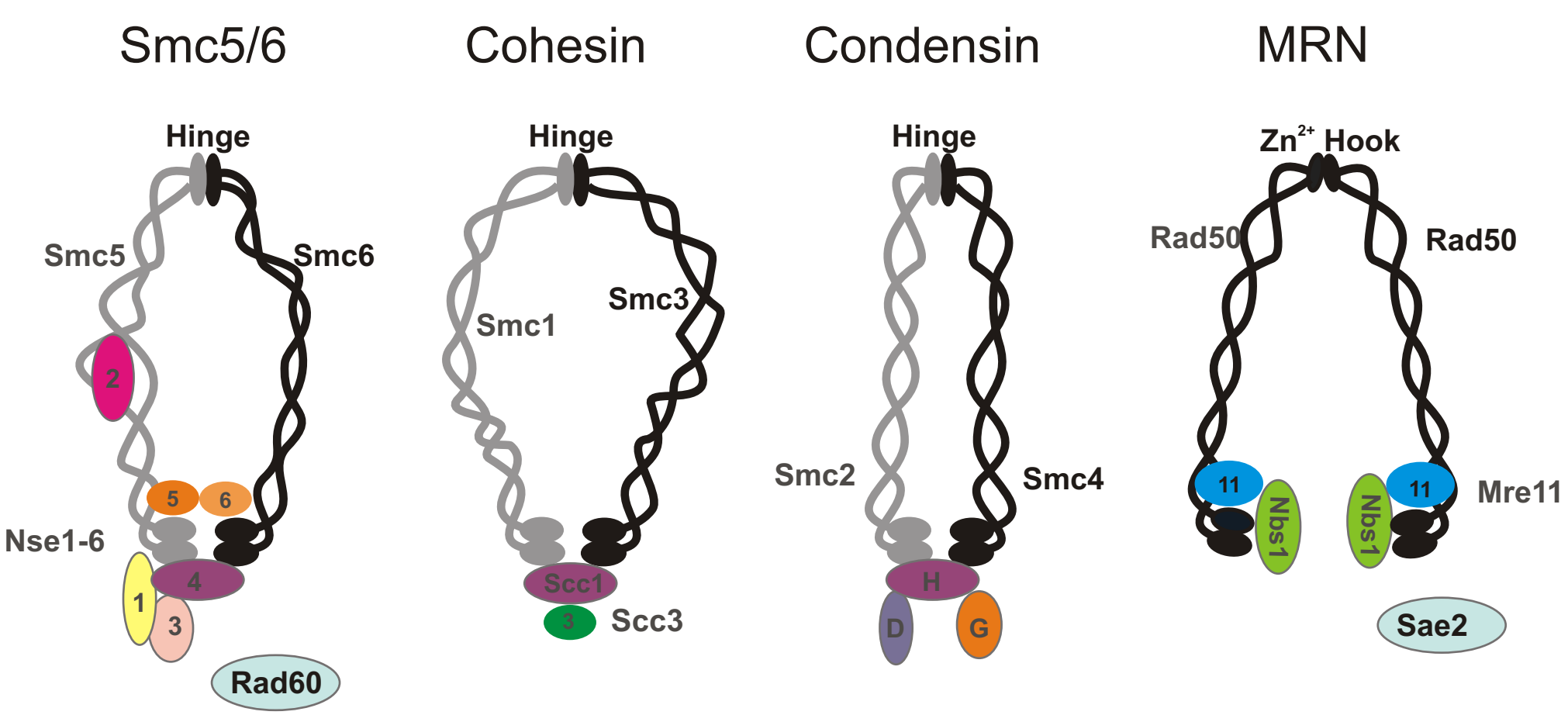
Cohesin has two non-SMC subunits, Scc1 and Scc3. Scc1 is a member of the kleisin family and bridges the heads of Smc1 and Smc3 to form a ring-like structure. Scc1 cleavage at the metaphase-anaphase transition by a specialized protease opens the ring and allows the sister chromatids to segregate. It is likely that the Smc1/3 ring encircles the two sister chromatids[39].

Yeasts have one Condensin complex, higher eukaryotes have two. Both share the same Smc2/4 subunits, but have evolutionarily diverged D, G and H subunits. H subunits are related to kleisins, bind the Smc2/4 head domains but are not cleaved. The D and G subunits contain HEAT repeats and associate via the kleisin subunit.

The MRN complex shares similar features, but the hinge region is replaced by a specialized interaction domain termed a ‘zinc hook’.

Nse1 resembles a RING-finger ubiquitin E3 ligase[24] but no in vitro ubiquitylation activity has been reported. Nse2 functions as an E3 SUMO ligase in vitro[22,24]. In vivo, several proteins have been identified as potential Nse2 substrates[22,24,25]. Nse3 has a MAGE (type II melanoma antigen) domain. Nse3 is the only MAGE domain protein in yeasts, although there are multiple MAGE domain proteins in higher eukaryotes. Nse4 resembles kleisins and may bridge the heads of Smc5 and Smc6[40]. Nse5 and Nse6 also associate with the head domains, potentially forming a second bridge[13].

Word count: 316


Smc5/6
Hinge
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Rad60
Cohesin
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Smc3
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Scc3
Condensin
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Smc4
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D
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MRN
Zn2+ Hook
Rad50
Rad50
11
Nbs1
Nbs1
11
Mre11
Sae2

Box 2. Alternative lengthening of telomeres (ALT) pathway.

Chromosome ends or telomeres comprise repetitive DNA elements bound by specific proteins (Shelterin components, comprising TRF1, TRF2, TIN2, TPP1, POT1 and RAP1[41]), which protect the end from degradation and prevent DNA damage signalling. In the absence of telomerase, telomeres shorten because normal replication cannot synthesise the last few nucleotides. This leads to cellular senescence. Normal somatic cells repress telomerase to limit proliferative potential. Telomerase is up regulated in many cancer cells to allow unlimited proliferation. In telomerase minus cancer cells, HR is used to increase telomere length (Alternative Lengthening of Telomeres - ALT - pathway). Characteristically, in cells undergoing ALT, telomeres associate with promyelocytic leukaemia PML bodies (ALT-associated PML bodies or APBs).

PML bodies are dynamic nuclear structures involved in numerous cellular processes. They facilitate post-translational modification and may localise proteins to sites of action. Many components of PML bodies are sumoylated [42]. In ALT cells, the Smc5/6 complex and HR proteins associate with PML bodies in G2 (this is when a sister chromatid is available). The SUMO-ligase activity of Nse2 is not required for Smc5/6 PML association. However, sumoylation of components of the Shelterin complex by Nse2 is required for the formation of APBs[25]. Thus, sumoylation of Shelterin recruits or maintains telomeres at APBs and promotes telomere HR.

Word count: 209

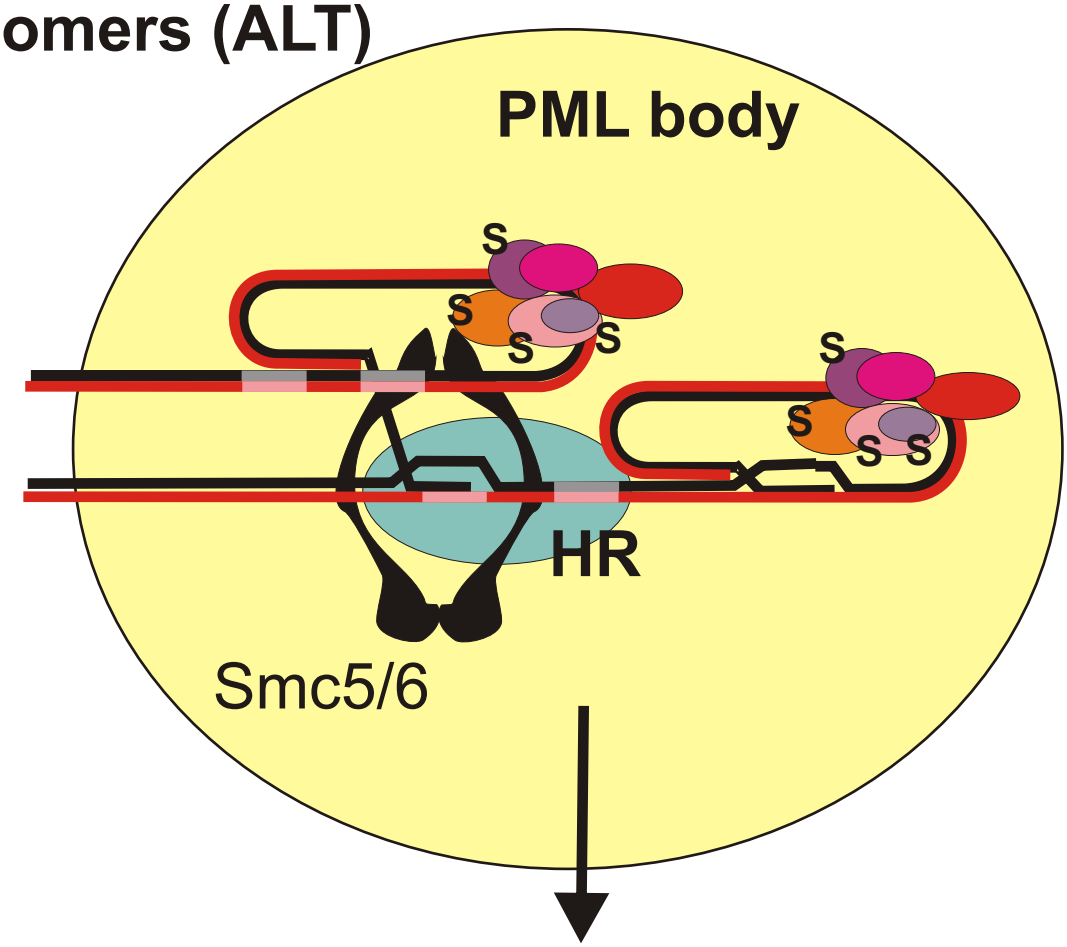
Telomerase Pathway

Shelterin

long telomere
telomerase blocked

short telomere
telomerase active

Alternative Lengthening of Telomers (ALT)



long telomere
extended replication potential

original telomere +

Figure 1. Unidirectional replication

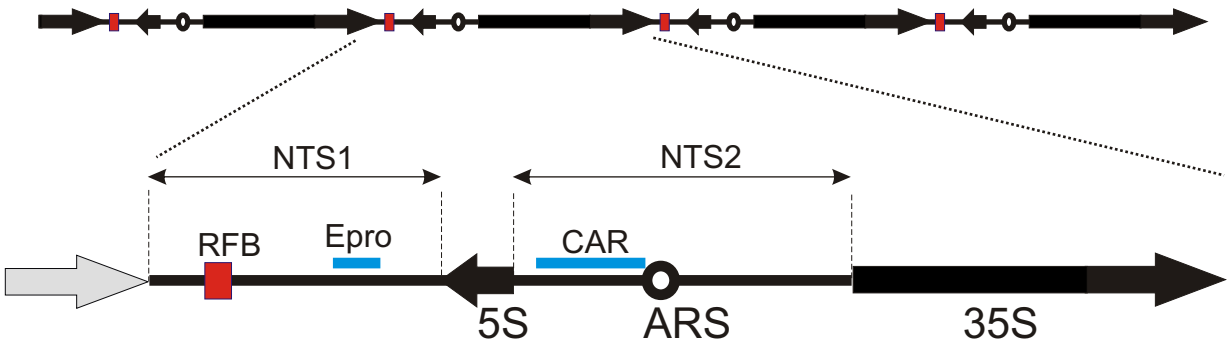
A. Details of the rDNA repeat are shown including the non-transcribed sequences (NTS1 and NTS2), the position of the RNA polymerase III (pol III) transcribed 5S and pol I transcribed 35S rDNA transcripts, the origin of replication (ARS) and replication fork barrier (RFB).

B. The Cohesin associate region (CAR) specifically recruits Cohesin to the rDNA while the pol II *epro* promoter is capable of dissociating Cohesin when activated. When the leftward travelling replication fork is arrested and broken by the polar RFB, Cohesin maintains sister chromatid alignment, ensuring HR results in equal sister chromatid exchange and repeat number remains stable (left panel). When *epro* transcription displaces cohesion (right panel) unequal sister chromatid exchange can occur, resulting in repeat amplification (shown) or loss (not shown).

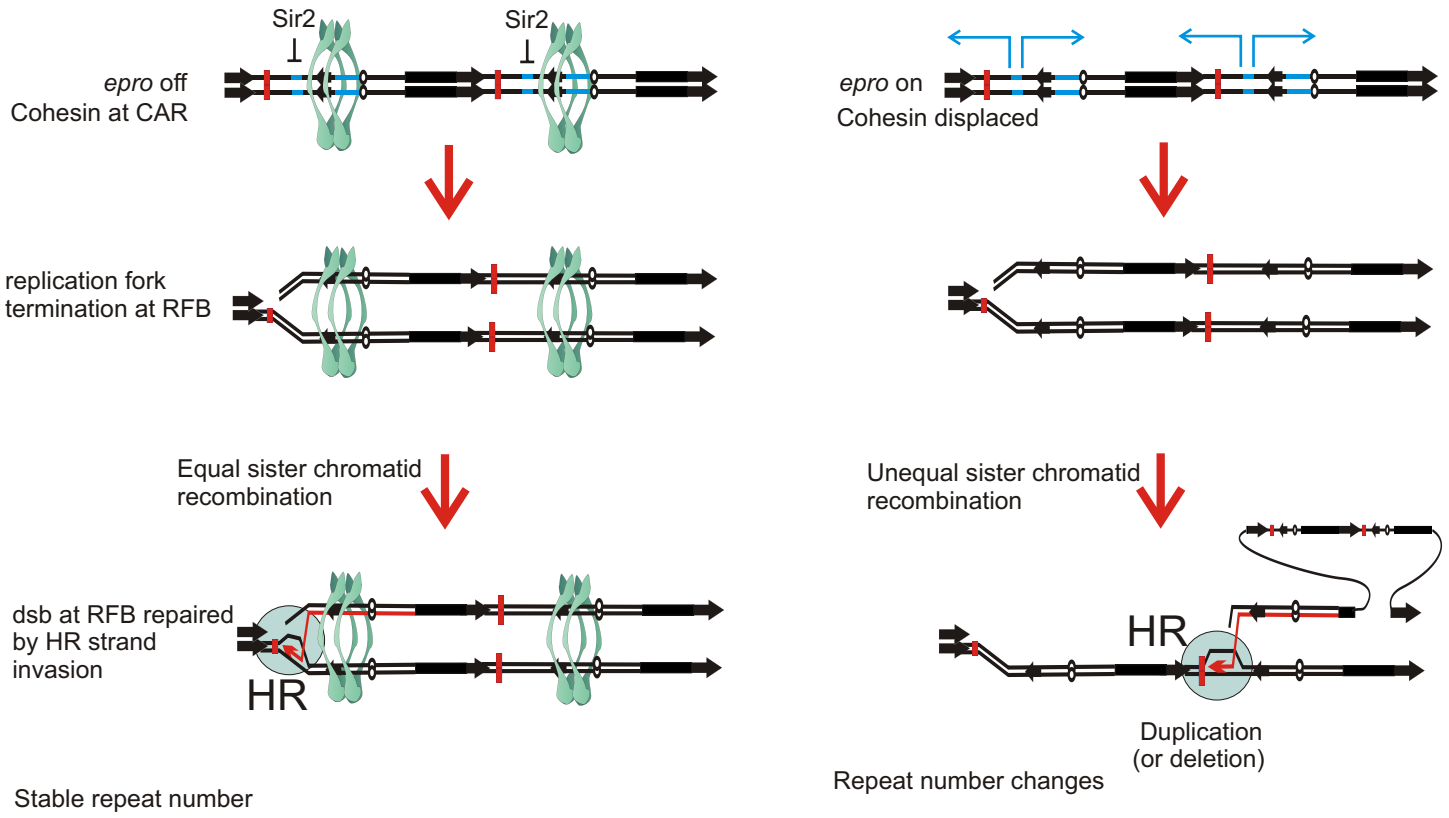
C. Schematic showing how a stopped fork (X) in a region of bidirectional replication can be rescued by a converging fork, whereas a similar situation in a region of unidirectional replication results in an unreplicated region. We use the terminology "stopped" to include stalled and collapsed forks that are not restarted. Most stalled forks can be reactivated and most collapsed forks can be rescued by HR. A stopped fork in a unidirectionally replicated region will have an absolute requirement for reactivation or rescue if an unreplicated region is going to be avoided. Thus, HR and its regulation are likely to become more important in such regions. Blue bar: RFB. Red arrow: direction of fork movement.

Word count: 239

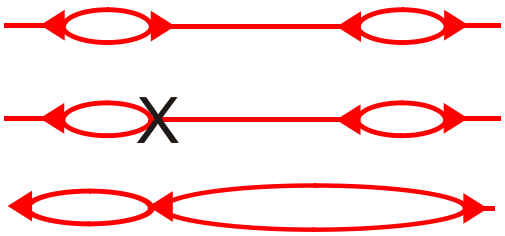
A. rDNA repeat unit



B. Cohesin association stabilises rDNA repeat number



C. Bidirectional replication



Unidirectional replication